Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing of Commercial Equine Tetanus Antitoxin by CELISA

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Contact Person: Donald R. Kolbe, (515) 663-7307

Approvals:

_____ Date:_____
Linda R. K. Schlater, Head
Biologics Bacteriology Section

_____ Date:_____
P. Frank Ross, Acting Quality Assurance Manager

_____ Date:_____
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for Potency Testing of Commercial Equine Tetanus Antitoxin by CELISA

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Supplemental Assay Method for Potency Testing of Commercial Equine Tetanus Antitoxin by CELISA

1. Introduction

This is a Supplemental Assay Method (SAM) Competitive Enzyme-Linked Immunosorbent Assay (CELISA) is used to determine the potency of commercial equine tetanus antitoxin. If the potency test is found to be unsatisfactory by the CELISA the product may be retested using the comparative toxin-antitoxin neutralization test in guinea pigs.

2. Materials

2.1 Equipment/insturmentation

2.1.1 Orbital mixer

2.1.2 Test tube mixer, vortex type

2.1.3 Refrigerated incubator (Hotpack, model 352600 or equivalent)

2.1.4 Refrigerator, 2°-7°C

2.1.5 Freezer, -20°C or lower

2.1.6 Screw-top glass Erlenmeyer flask, 250 ml

2.1.7 Screw-top glass Erlenmeyer flask, 500 ml

2.1.8 Screw-top glass Erlenmeyer flask, 1 L

2.1.9 Screw-top glass bottles, 1 L

2.1.10 Automatic microplate washer/aspirator

2.1.11 Microplate reader

2.1.12 Personal computer (PC) with software package for data analysis

2.1.13 Flat-bottom 96-well microtitration plates (Nunc™ F96-well maxisorb or equivalent)

2.1.14 Micropipettes, 100 µl and 1000 µl

2.1.15 Tips for 100 µl and 1000 µl micropipettes

2.1.16 Multichannel micropipette, 50 to 300 µl

2.1.17 Tips for 50 to 300-µl multichannel micropipette

2.1.18 Pipettes, 5 ml, 10 ml, and 25 ml

2.1.19 Screw-top polystyrene tubes, 17 x 120 mm, with caps

2.1.20 Screw-top polypropylene tubes, 50 ml, with caps

2.1.21 Reagent trays

2.1.22 Bulb-type safety pipette filler

2.1.23 Standard class A 10-ml graduated cylinder

2.1.24 Standard class A 50-ml graduated cylinder

2.2 Reagents

2.2.1 Water, deionized or distilled, or water of equivalent purity

2.2.2 Sodium chloride (NaCl)

2.2.3 Sodium bicarbonate ($NaHCO_3$)

2.2.4 Sodium carbonate (Na_2CO_3)

2.2.5 Sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2.2.6 Sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

2.2.7 Sodium hydroxide (NaOH)

2.2.8 Polyoxyethylene-sorbitan monolaurate (Tween 20)

2.2.9 Ammonium thiocyanate (NH_4SCN)

2.2.10 Tetramethylbenzadine (TMB) peroxidase substrate system

2.2.11 Hydrochloric acid (HCl)

2.2.12 Sodium hydroxide (NaOH)

2.2.13 Sulfuric acid (H_2SO_4)

2.2.14 Positive horse serum

2.2.15 Negative horse serum

2.2.16 *Clostridium tetani* toxin

2.2.17 Tetanus monoclonal antibody conjugated with horseradish peroxidase

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need working knowledge of the use of general laboratory chemicals, equipment, and glassware. They need specific training and experience in the safe handling of *Clostridial* toxins and performing CELISA.

3.2 Preparation of equipment/instrumentation

Operate all equipment according to the manufactures' instruction.

3.3 Preparation of reagents/control procedures

3.3.1 Wash solution

1. Add 8.5 g of NaCl, 0.22 g $NaH_2PO_4 \cdot H_2O$, 1.19 g Na_2HPO_4, and 0.5 ml Tween 20 to 1 L of water. Heat in a boiling water bath to dissolve completely.

2. Adjust the pH to 7.2 with 5 M NaOH if necessary. Dispense approximately 850 ml of wash solution into 1-L bottles and autoclave at 121°C for 30 min with caps loosened. After the containers have cooled tighten the caps and store at room temperature.

3.3.2 Antigen coating buffer

1. Add 0.80 g Na_2CO_3, and 1.45 g $NaHCO_3$ to 500 ml of water. Mix until completely dissolved.

2. Adjust the pH to 9.6 with 5 M NaOH if necessary.

3. Store the buffer at 2°-7°C in a 500-ml Erlenmeyer screw-cap flask for no longer than 1 wk.

3.3.3 Blocking buffer

1. Add 0.1 g $NaH_2PO_4 \cdot H_2O$, 1.9 g Na_2HPO_4, and 20 g of NaCl to 1 L of water. Heat in a boiling water bath to dissolve completely.

2. Adjust the pH to 7.9 with 5 M NaOH.

3. Store the buffer at 2°-7°C in a 1-L Erlenmeyer flask for no longer than 2 wk.

3.3.4 Serum diluting buffer (SDB)

1. Add 175 ml of 100 mM Na_2HPO_4 (14.2 g per liter) to 1 L of 100 mM $NaH_2PO_4 \cdot H_20$(13.9 g per liter).

2. Add 3.8 g of NH_4SCN to 1 L of 100 mM phosphate buffer and mix until dissolved.

3. Adjust the pH to 6.0 with 10% HCl if necessary.

4. Store the buffer at 2°-7°C for no longer than 1 wk.

CAUTION! Toxic and Hazard Review (THR) of ammonium thiocyanate: Poison by ingestion. Moderately toxic by other routes. Human systemic effects by ingestion: hallucination and distorted perceptions, nausea or vomiting, and other gastrointestinal effects. When heated to decomposition it emits toxic fumes of NH_3, NO_x, SO_x and CN^-.

3.3.5 Conjugate diluent

1. Add 0.3 g $NaH_2PO_4 \bullet H_2O$, 1.1 g Na_2HPO_4 and 20 g NaCl to 1 L of water.

2. Heat in a boiling water bath to dissolve completely.

3. Adjust the pH to 7.2 with 5 M NaOH.

4. Store the buffer at 2°-7°C for no longer than 2 wk.

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3.3.6 Tetanus toxin

Coat the surface of the microtitration plate with *Clostridium tetani* toxin obtained from the United States Department of Agriculture (USDA), Veterinary Services (VS), Center for Veterinary Biologics-Laboratory (CVB-L).

CAUTION! Accidental parenteral inoculation and ingestion of tetanus toxin are the primary hazards to laboratory personnel. It is unknown if the toxin can be absorbed through mucous membranes, consequently, the hazards associated with aerosols and droplets remain unclear.

3.3.7 Negative horse serum

Block the microtitration plate with negative horse serum obtained from the CVB-L. The serum has an absorbance value of approximately 1.5 when tested by CELISA and is used as a zero titer.

3.3.8 Positive horse serum

Use positive horse serum obtained from the CVB-L containing 500 American units of tetanus antitoxin per ml to prepare the positive controls.

3.3.9 Conjugate

Use tetanus monoclonal antibody (MAB) obtained from the CVB-L to perform the CELISA. The MAB is conjugated to horseradish peroxidase and identified as IRP 439.

3.3.10 Substrate

Use TMB peroxidase substrate system which develops a deep blue product when reacted with peroxidase labeled conjugates, and when stopped with H_2SO_4 becomes yellow. The substrate may be purchased from Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879.

3.3.11 2.5 M H_2SO_4 (Stop Solution)

1. Add 27.2 ml of H_2SO_4 to 150 ml of water. Q.S. to 200 ml with water.

2. Dispense into 250-ml screw-top Erlenmyer flasks and store at room temperature.

CAUTION! Toxic and Hazard Review (THR) of sulfuric acid: Human poison by unspecified route. Experimental poison by inhalation. Moderately toxic by ingestion. A severe eye irritant. Extremely irritating, corrosive, and toxic to tissue resulting in rapid destruction of tissue, causing severe burns. If much of the skin is involved, it is accompanied by shock, collapse and symptoms similar to those seen in severe burns. There are systemic effects secondary to tissue damage caused by contact with it. However, repeated contact with dilute solutions can cause a dermatitis, and repeated or prolonged inhalation of a mist of sulfuric acid can cause inflammation of the upper respiratory tract leading to chronic bronchitis.

3.3.12 5 M NaOH

1. Add 200 g of NaOH pellets to a 500-ml screw-top Erlenmeyer flask.

2. Add 200 ml of water to the flask and allow the pellets to dissolve overnight.

3. Store at room temperature.

CAUTION! Toxic and Hazard Review (THR) of liquid sodium hydroxide: Poison by intraperitoneal route. Moderately toxic by ingestion. Mutagenic data. A corrosive irritant to skin, eyes, and mucous membranes. This material, both solid and in solution, has markedly corrosive action upon all body tissue causing burns and frequently deep ulceration, with ultimate scarring. Mist, vapors, and dusts of the compound cause small burns, and contact with the eyes rapidly causes severe damage to the delicate tissue.

3.3.13 10% HCl

1. Add 20 ml of hydrochloric acid to 160 ml of water. Q.S. to 200 ml with water.

2. Dispense into a 250-ml screw-top Erlenmyer flasks and store at room temperature.

CAUTION! Toxic and Hazard Review (THR) of hydrochloric acid: A human poison by an unspecified route. Midly toxic to humans by inhalation. Moderately toxic experimentally by ingestion. A corrosive irritant to the skin, eyes and mucous membranes. Mutagenic data. An experimental teratogen. A concentration of 35 ppm causes irritation of the throat after short exposure. In general, hydrochloric acid causes little trouble in industry other than from accidental splashes and burns.

4. Performance of the test

4.1 Measurement of equine tetanus antitoxin in final container

4.1.1 Check the container label for each product to be tested.

4.1.2 Determine the volume of antitoxin by inverting the opened container over a graduated volumetric cylinder until the flow stops. Use 10-ml cylinders to measure amounts of 10 ml or less, and 50-ml cylinders to measure amounts of 10.1 ml to 50 ml. Use graduated volumetric cylinders that conform to the National Bureau of Standards requirements.

4.1.3 Record the volume of antitoxin in the cylinder by taking the reading at the bottom of the meniscus to the nearest 0.1 ml for the 10-ml cylinder, and to the nearest 1.0 ml for the 50-ml cylinder.

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4.2 Potency requirements for equine tetanus antitoxin

4.2.1 All final containers of tetanus antitoxin shall yield not less than the labeled unitage of antitoxin throughout the dating period. The minimum package size permitted for marketing in the United States is a 1,500 unit vial.

4.2.2 When testing is done on bulk material, the final container fill shall reflect the endpoint value plus 10% overage for a 1 yr dating and 20% overage for a 3 yr dating.

4.3 CELISA procedure

4.3.1 Coating the microtiter plate with tetanus toxin

1. Dilute *Clostridium tetani* toxin IRP 433 1:15 in antigen coating buffer.

2. Dispense 100 µl of diluted toxin into each well of a 96-well microtitration plate. Cover the plate with a lid and place it in a humid chamber. Incubate the plate overnight at 20°-25°C on an orbital shaker operating at approximately 120 revolutions per min.

3. At the end of the incubation period remove the lid and shake the unbound toxin into an autoclavable container.

4.3.2 Blocking the microtitration plate

1. Prepare a 1:100 dilution of Negative Equine Serum IRP 429 by adding 0.1 ml of IRP 429 to 9.9 ml of blocking buffer. Further dilute the serum to 1:2,000 by adding 1.0 ml of the 1:100 dilution to 19 ml of buffer.

2. Dispense 200 µl of IRP 429 diluted 1:2,000 into each well of the microtitration plate. Cover the plate and incubate it at 35°-37°C in a humid chamber for 120-130 min while mixing on an orbital shaker. (Note: Use an incubator with refrigeration capability or the heat from the shaker will cause the temperature to increase to an unacceptable level.)

3. At the end of the incubation period wash each well 5 times using approximately 0.3 ml of wash solution each time. Invert the plate and tap it on absorbent material to remove all traces of residual wash solution.

4. Invert the covered plate and store in a sealed plastic bag at 2°-7°C. Use the plate the same day it is blocked with negative horse serum.

4.3.3 Serum sample dilutions

1. Dilute positive horse serum IRP 445 1:6000 in SDB to generate a 500 AU per ml standard within the working sensitivity range of the CELISA. From this, incremental dilutions of 4:1, 3:2, 2:3, and 1:4 are made to generate standard samples corresponding to 400, 300, 200, and 100 AU per ml (Fig. 1).

2. Dilute normal horse serum 1:6000 to generate a 0 AU standard (Fig. 1).

3. Dilute the unknown antitoxin to be tested in a manner similar to the positive horse serum, to generate 3 dilutions per serum sample (Fig. 2).

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Figure 1. Dilutions of Positive and Negative Horse Serum

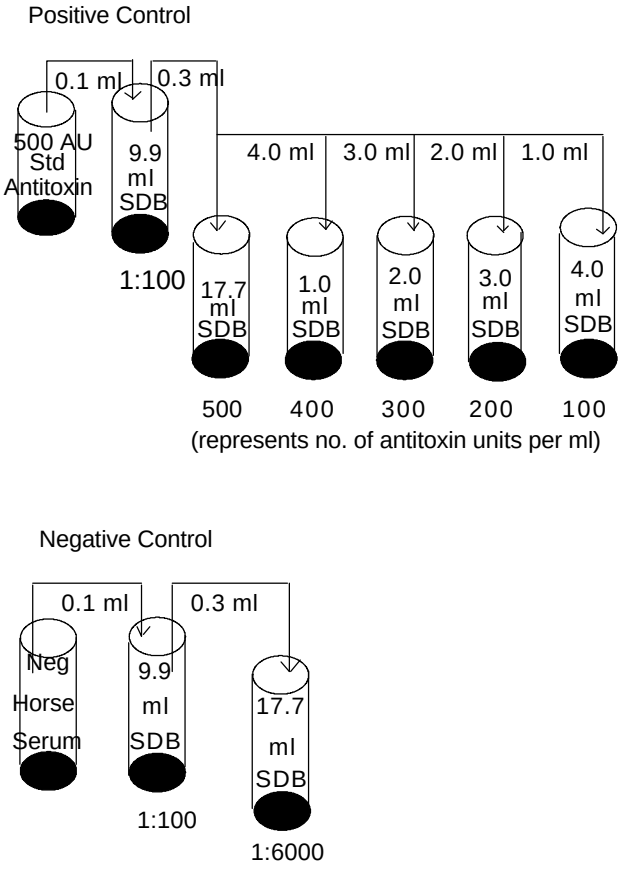

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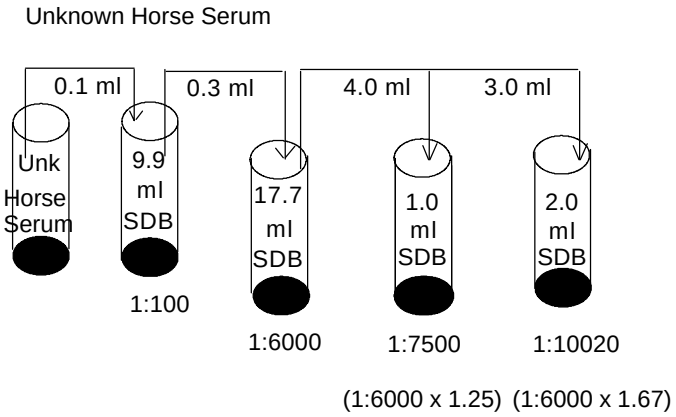
Figure 2. Dilutions of Unknown Horse Serum



4. Add 100 µl of diluted negative and positive horse serum samples to each of 6 wells in columns 2 through 7 (Fig. 3).

5. Add 100 µl of diluted test serum samples to each of 6 wells in colums 8 through 10.

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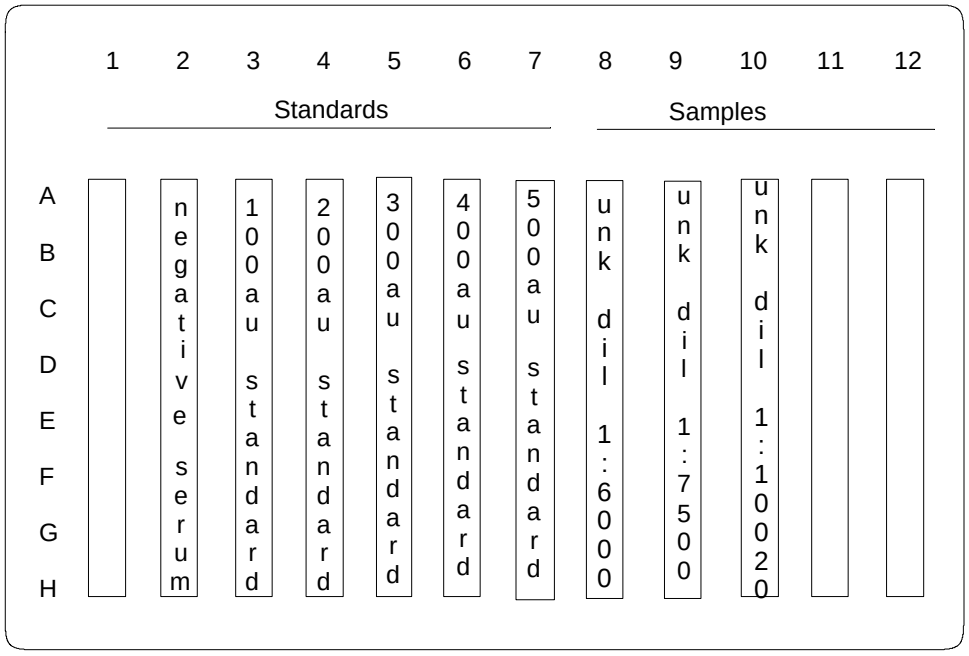
Fig. 3 Test Format for Equine Tetanus CELISA



6. Cover the plate and incubate it at 20°-25°C in a humidified chamber for 20-22 hr.

4.3.4 Addition of conjugate

1. Wash the plate 5 times as previously described.

2. Dilute the conjugate 1:14,000 by adding 20 µl of conjugate to 19.98 ml of conjugate diluent. Further dilute the conjugate by adding 1.0 ml of the 1:1000 dilution to 13 ml of diluent.

3. Add 100 µl of the diluted conjugate to each well of the plate.

4. Cover the plate and incubate at 35°-37°C in a humid chamber for 90-100 min while mixing in a humidified chamber.

4.3.5 Addition of substrate

1. Remove the plate from the incubator and wash 5 times as previously described.

2. Dispense 100 µl of TMB peroxidase substrate solution into the wells of the plate.

3. Cover the plate and incubate it at 20°-24°C for 9-10 min while being mixed.

4.3.6 Addition of stop solution

1. Stop the colorimetric reaction by adding 100 µl of stop solution to each well of the plate.

2. Place the microtiter plate on a rotary shaker and mix for approximately 5 min before measuring the absorbance readings.

4.3.7 Absorbance readings

Measure the absorbance of the CELISA plate in a microplate reader, with a T setting of 450 nm and a R setting of 650 nm, coupled to a PC containing software for data analysis.

4.4 Calculation of results

4.4.1 A best-fit linear regression line is calculated using the mean absorbance value for the standard serum dilutions (Fig. 4). The mean absorbance values of the unknown serum samples are entered into the equation of the standard serum regression line to estimate the antitoxin titers of the unknown samples.
Estimates of antitoxin titers derived from individual dilutions of the unknown sample are averaged to derive a mean estimate of AU per ml.

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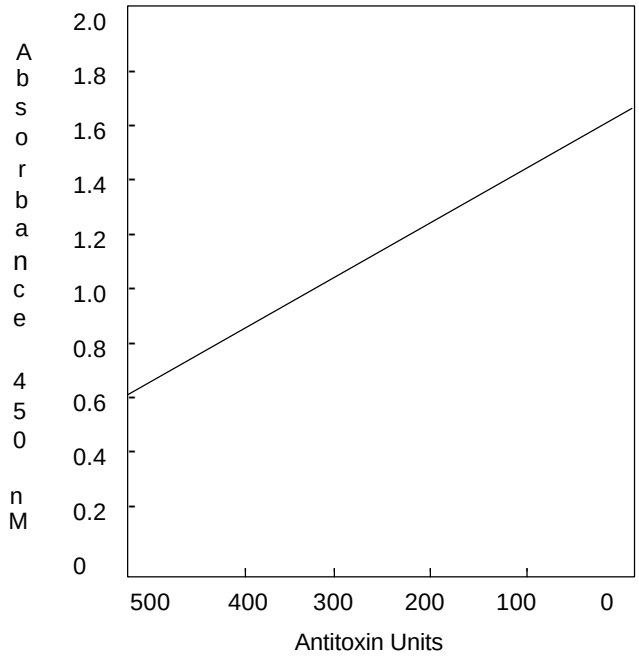
Fig. 4 Tetanus Antitoxin Standard Curve 500 AU to Negative



4.4.2 Formula used to determine the slope and correlation coefficient (r)

y = OD
x = antitoxin units
n = antitoxin levels

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$$slope = \frac{\sum xy - \frac{(\sum x)(\sum y)}{n}}{\sum x^2 - \frac{(\sum x^2)}{n}}$$

$$y - intercept = \frac{\sum y}{n} - slope\ (\frac{\sum x}{n})$$

$$correlation\ (r)\ \frac{\sum xy - \frac{(\sum x)(\sum y)}{n}}{\sqrt{\sum y^2 - \frac{(\sum y)^2}{n}}\ x\ \sqrt{\sum x^2 - \frac{(\sum x)^2}{n}}}$$

$$\text{Antitoxin units} = \frac{\text{unknown OD - y intercept}}{\text{slope}}$$

5. Interpretation of test results

5.1 The best reproducibility is obtained when the mean absorbance of the 0 AU per ml standard is 1.4-1.6 and the mean absorbance of the 500 AU per ml standard is 0.4-0.6. Tests with correlation coefficients less than 0.990 are considered NO TESTS and may be repeated.

5.2 Dilutions of the unknown serum samples with absorbance values of 0.4-1.0 are used to determine the average number of AU per ml. Dilutions with absorbance readings outside of this range are less accurate for estimating antitoxin titer. If the absorbance value of all 3 dilutions of an unknown sample are below 0.4, the test is considered invalid. The sample should be retested at twofold greater dilutions, and estimations of titer from readings falling in the 0.4-1.0 range multiplied by 2. If the mean absorbance values of all 3 dilutions are above 1.0, the test is also invalid. The dilution factor of serum sample should be reduced twofold, and the antitoxin readings calculated from dilutions with absorbance values of 0.4-1.0 divided by 2.

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6. Reporting of the test results

Results of the test(s) are reported as described by standard Section operating procedures.

7. References

Code of Federal Regulations, Title 9, Part 113, U.S. Government Printing Office, Washington, DC, 1999.

8. Summary of revisions

This document was rewritten to meet the current CVB/NVSL QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. Significant changes were made from the previous protocol.